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Concerning the Anomalous Kinetic Behavior of Microtubules*

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We have demonstrated that tubulin-GTP subunits can react with microtubule ends containing subunits with E-site-bound GDP. This observation can be taken to rule out a previous interpretation of a biphasic dependence of the rate for subunit flux into microtubules on the subunit concentration, which is based upon an assumption that GTP is required to be present in subunits at microtubule ends in order to allow addition of tubulin-GTP subunits. The nullified mechanism had been suggested to be the basis of the observation that growing and shrinking microtubules coexist as independent species. We have also confirmed previous studies indicating that the flux rate is nonlinearly dependent on the subunit concentration and account for this behavior by assuming that tubulin-GTP subunits reversibly add to microtubule ends by two paths. In one, tubulin-GTP subunits add nonproductively to generate an end which is unable to undergo further net microtubule elongation; however, this reaction can retard the rate for microtubule disassembly under conditions where the disassembly reaction predominates. In the other, tubulin-GTP subunits add productively to microtubule ends to generate ends which can undergo subsequent net elongation.

It has been reported (1) that "microtubules *in vitro* coexist in growing and shrinking populations which interconvert infrequently." This unusual behavior was accounted for by a mechanism (2, 3) in which: (*a*) microtubules have a "cap" of TuT[1] subunits at each end of a much longer core of TuD subunits; (*b*) the dissociation rate for TuT subunits is very much less than for TuD subunits, so that at steady capped microtubules are stable (and can undergo net growth) until a stochastic fluctuation in the length of the TuT cap exposes the TuD core; and (*c*) TuT subunits do not add to microtubule ends containing TuD subunits (TuT must be at an end to allow subunit addition). Premise *c* predicts that microtubules which lose their TuT cap constitute a monotonically shrinking population, liberating subunits which add to the ends of a separate (capped) population of microtubules which undergoes net growth. The observed *in vitro* results (1) have been successfully simulated in a computer analysis (4) based upon this model.

The basic premise of the proposed model, that TuD subunits do not add to uncapped ends (*c*, above), is derived from studies of the effect of GDP on the pre-steady-state microtubule elongation rate (2, 5). It was found (2, 5) that added GDP decreases this rate by reducing the TuT subunit concentration *and* by modifying the reactivity of microtubule ends. This effect of GDP on microtubule ends was presumed to result from an exchange of GDP into the E-site of tubulin subunits at the tip of the microtubule (*i.e.* the κ'' path in Fig. 1 in Ref. 6[2]). In essence, GDP binds to and modifies an end by altering the conformation of terminal subunits. However, an alternate path, which also accounts for the GDP effects, was noted (see Equation 8 in Ref. 2) in which TuD subunits add to an end in a manner such that subsequent TuT subunit addition is precluded. We have recently developed a model based upon this alternate path for GDP inhibition (7) in which we consider the kinetic consequences for nonproductive addition of TuD as well as TuT subunits to microtubule ends. Nonproductive refers to the fact that subsequent TuT subunit addition is precluded. This alternate model for accounting for GDP inhibition of microtubule elongation differs in an important way from the proposed scheme (2, 3) in which inhibition results from GDP exchange into terminal subunits: it allows for TuT subunit elongation of microtubule ends containing *productively* bound TuD subunits. We had previously described a mechanism (page 12056 in Ref. 8) in which microtubule ends can contain two kinds of TuD subunits, productively bound TuD subunits introduced into the microtubule's core in a reaction which is accompanied by GTP hydrolysis, and nonproductively bound TuD subunits incorporated into an end by TuD subunit addition from solution. It is presumed that ends containing productively bound TuD subunits will allow TuT subunit addition.

The just-described mechanism is inconsistent with premise *c* of the model proposed by Carlier and co-workers (2, 3, 6). Differentiating between the models requires determining whether TuT subunits can react with the core of TuD subunits derived from the microtubule's interior. Study of such a reaction has been found to be feasible because the nucleotide in TuT subunits exchanges with GDP relatively slowly (half-time of about 30 s (9)), and the rate for dilution-induced disassembly of MAP-free microtubules is sufficiently rapid that TuT can react before the GTP dissociates from the tubulin dimer. These two factors have allowed a study of the reaction of TuT subunits with disassembling microtubule ends under conditions where the ends can only contain GDP. Our results indicate that TuT subunits react with microtubule ends containing TuD subunits. From this, we conclude that

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[1] The abbreviations used are: TuT, tubulin subunits containing E-site GTP; TuD, tubulin subunits containing E-site GDP; MAP, microtubule-associated protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

[2] The rate constant nomenclature is from Ref. 6: κ'' is for the exchange of subunit-bound GDP with unbound GTP in subunits at the microtubule's tip; α_{1T} and α_{-1T} describe TuT subunit addition to and loss from an end which has GTP-containing subunits; α_{1D} is for TuT addition to an end which has GDP-containing subunits; α_{-2D} and α_{2D} describe TuD subunit gain at and loss from an end which had GDP-containing subunits.

the model which was proposed (1) to account for the dynamic instability of microtubules is apparently incorrect.

EXPERIMENTAL PROCEDURES

Materials—Microtubular protein was purified from pig brain by cycles of thermal-induced assembly (10), using a reassembly buffer consisting of 0.1 M MES, 0.5 mM $MgCl_2$, 1.0 mM EGTA, pH 6.80. Pellets obtained from this procedure (stored at −80 °C) were defrosted and, after another assembly/disassembly cycle, the protein was desalted on Sephadex G-25. Non-tubulin proteins were then removed on Sepharose 6B, and the broad, relatively dilute tubulin dimer peak was concentrated with an Amicon YM-10 membrane. This material is stable for at least 1 month when stored at −80 °C. Prior to use, the protein was centrifuged (Airfuge, 8 p.s.i., 2 min) to remove aggregated material. Microtubules formed with this protein (in a dimethyl sulfoxide-induced assembly (11), under the conditions described below) and isolated by high speed centrifugation were found (12) to contain less than 1% non-tubulin proteins. When the pelleted polymer was sectioned and examined microscopically, microtubules and C- and S-shaped polymers were present in an approximately 9:1 ratio.

Microtubules used in kinetic studies were assembled to a steady state by a 1-h incubation with dimethyl sulfoxide (12%), $MgCl_2$ (1.5 mM final concentration), acetate kinase (0.075 unit/ml), acetyl phosphate (11 mM), and a trace amount of high specific activity [^{3}H]GTP. The acetyl phosphate and acetate kinase concentrations were reduced 24- and 14-fold, respectively, when the microtubules were used in studies in which microtubules were diluted 25-fold into 200 μM GDP or GTP, or into TuD. When the GTP-regenerating system is so diluted, the lag preceding microtubule assembly is a bit longer, but there is no measurable effect on the amount of microtubule assembly at steady state.

The TuD used for dilution and for assembly studies was prepared by incubating 20 μM tubulin for 20 min at 37 °C with phosphofructokinase (EC 2.7.1.11) (22 unit/ml) and fructose 6-phosphate (0.75 mM). In control reactions, using the analytical method previously described (13), it was found that tubulin-bound [^{3}H]GTP is 80–90% converted to [^{3}H]GDP in 1 min under these conditions.

Acetate kinase (EC 2.7.2.1) (Sigma type A 2384) was desalted by column centrifugation (14) and phosphofructokinase (Sigma type F 6877) was depleted of accompanying salt and nucleotides by dissolving the material obtained by centrifugation (maximum speed for 10 min in an Airfuge) in reassembly buffer.

Methods—Kinetic studies were carried out at 37 °C, and the buffer used for dilution studies was equilibrated to this temperature before use. The number of moles of guanine nucleotide incorporated into microtubules was determined by measuring the amount of radioactivity which could be extracted (with perchloric acid) from microtubules assembled in the presence of [^{3}H]GTP and then subjected to centrifugation (3 min at maximum speed in an Airfuge). The amount of radioactivity per mole of tubulin incorporated into microtubules depends upon the stoichiometry for tubulin nucleotide binding and the specific radioactivity of the [^{3}H]GTP in the assembly. Two variables determine the specific radioactivity, the amount of added GTP, and the amount of GDP and GTP provided by the tubulin E-site. With regard to the latter, this is not necessarily equal to the tubulin concentration, since GTP and GDP may be lost from the E-site during protein purification. Since two variables influence the specific radioactivity, it is possible to determine this parameter (and, thereby, the stoichiometry for [^{3}H]GXP incorporation into microtubules) by measurement of label incorporation at at least two concentrations of added GTP. For example, if label uptake in assembly with 30 μM tubulin and a trace amount of [^{3}H]GTP is reduced by a factor of two by adding 30 μM GTP, then the 30 μM tubulin contains 30 μM E-site-bound and unbound GTP (and GDP, since this is converted to GTP by the acetate kinase). From one additional measurement at a different concentration of added GTP, the number of moles of [^{3}H]GXP incorporated into microtubules can be determined. This titration procedure was also used to estimate the critical concentration for TuT subunits. In this case, the number of moles of tubulin-[^{3}H]GXP in pelleted microtubules was determined after microtubules were diluted; the critical concentration was taken to be equal to the decrement in sedimentable [^{3}H]GXP per increment in volume. The advantage of this method is that it allows the critical concentration to be expressed in terms of active TuT subunits. Purified tubulin contains significant amounts of inactive protein (32% of a purified tubulin sample was found to be inactive in a recent study (15)) so that the critical concentration cannot be accurately determined by measurement of the protein concentration of the supernatant derived from centrifugation of microtubules at steady state.

Rates for dilution-induced microtubule disassembly following a 25-fold dilution were determined by diluting 10 μl of radioactive microtubules into 240 μl of buffer. Reactions were quenched by addition of 7 ml of a solution containing reassembly buffer:glycerol:dimethyl sulfoxide (52:36:12). Prior to microtubule isolation (centrifugation in a Beckman Ti-50 rotor, 30 °C, 50,000 R.P.M., 20 min), 200 μl of a solution containing microtubules (assembled from the tubulin oligomer peak derived from the Sepharose 6B purification of tubulin (see above)) were added as carrier. The yield of pelleted radioactive nucleotide obtained by this method is equal to that obtained by centrifugation of undiluted samples in an Airfuge. Radioactive guanine nucleotide is only slowly lost from microtubules in quenched reaction mixtures; this rate was less than 2%/h over the course of 10 h. In studies in which the kinetics for microtubule disassembly was measured following a 2,500-fold dilution, 2 μl of radioactive microtubules were diluted into 5 ml of buffer containing 0.5% dimethyl sulfoxide, and the reactions were quenched by addition of 1.76 ml of glycerol and 0.92 ml of dimethyl sulfoxide.

RESULTS

Critical Concentration of Tubulin-GTP Subunits—We have used a titration procedure for determining the amount of [^{3}H]GXP incorporated into microtubules assembled in the presence of [^{3}H]GTP (Experiments I–III, Table I). For the representative experiment presented here, assembly of 20.5 μM tubulin yielded an amount of [^{3}H]GXP in microtubules corresponding to 11.8–12.9 μM in the original reaction mixture. The amount of pelleted protein corresponded to 16.4 μM, so that the stoichiometry for [^{3}H]GXP incorporation into microtubules is about 0.75. To determine the critical concentration for TuT in the presence of 12% dimethyl sulfoxide, microtubules which had been assembled to steady state in the presence of [^{3}H]GTP were diluted, and after a new steady state was attained, the microtubules were isolated by centrifugation. As indicated in Table I (Experiments IV and V), the amount of microtubule disassembly depends upon the extent of the dilution. From measurement of the effect of dilution on the amount of isolable microtubules, a critical concentration equal to 1.22–1.40 μM is estimated.

The TuT critical concentration is considerably higher in

TABLE I

Titration of [^{3}H]GXP incorporation into microtubules and determination of the subunit critical concentration

Microtubules were assembled to steady state in the presence of [^{3}H]GTP (8.06×10^4 cpm) and these were isolated by centrifugation in an Airfuge (3 min at maximum speed, room temperature); for Reactions IV and V, the microtubules were diluted to the indicated concentrations 10 min prior to centrifugation.

	Tubulin conc[a]	Added GTP	Radioactivity in pellet[b]	Endogenous GTP[c]	[^{3}H]GXP in microtubules[c]	Calculated critical conc
	μM	μM	cpm × 10^{-3}	μM	μM	μM
I	20.5	0	44.50 ± 0.50			
II	20.5	16.2	26.27 ± 0.47	23.25	12.9	
III	20.5	32.3	17.71 ± 0.23	21.29	11.8	
IV	10.25	0	19.25 ± 0.07[d]		10.95	1.40[e]
V	5.13	0	7.78 ± 0.24[d]		8.68	1.22[e]

[a] Concentrations were estimated from the absorbance at 278 nm (ε = 132,000).

[b] Mean of values observed in triplicate determinations.

[c] Concentrations correspond to those present prior to centrifugation; these were calculated from: radioactivity in pellet = (GXP in microtubules)(cpm added)/((endogenous GTP) + added GTP).

[d] These values are not corrected for dilution.

[e] Calculated from: ((mean value for GXP in microtubules for Reactions II and III) − (observed GXP in microtubules))/(increment in volume).

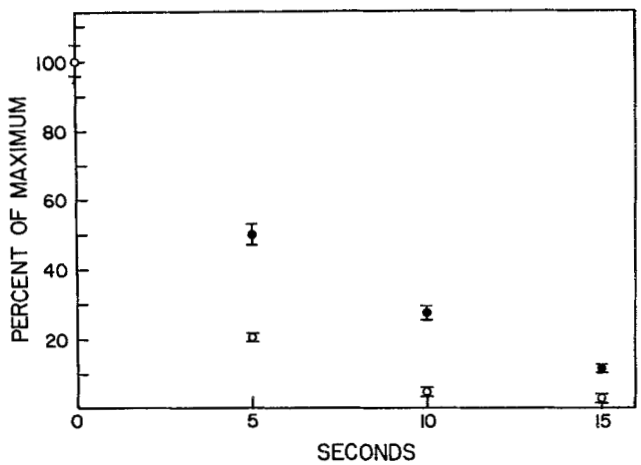

FIG. 1. **Dilution-induced disassembly of microtubules in buffer containing 0.5% dimethyl sulfoxide.** Microtubules were diluted 25-fold (●) and 2500-fold (○); the range for triplicate determinations at each time point is shown by the *bars*.

the presence of 0.5% dimethyl sulfoxide, and we are only able to provide a lower limit estimate for this value. This was determined by nucleating a reaction mixture containing 16.6 μM tubulin, an acetate kinase: GTP-regenerating system, and [^{3}H]GTP, with a 4% volume of microtubules, which had been assembled with acetate kinase and 12% dimethyl sulfoxide and then sheared by rapid passage through a syringe needle. Centrifugation of these samples after 30 or 60 min (3 min at maximum speed in an Airfuge) yielded a pellet containing 5% as much radioactive guanine nucleotide as was found in an identical sample with 12% dimethyl sulfoxide. Since with 12% dimethyl sulfoxide about 12 μM tubulin is incorporated into microtubules (Table I), it can be concluded that the critical concentration for TuT is at least 10 μM in the presence of 0.5% dimethyl sulfoxide, *i.e.* total active tubulin (12 + 1.4) − tubulin in microtubules (0.05 × 12).

Rate for Disassembly of Microtubules—The rate for the dilution-induced disassembly of MAP-free microtubules is extremely rapid in the presence of 0.5% dimethyl sulfoxide (Fig. 1 and Table II, Experiment Ib).[3] For the reaction shown here, the mean microtubule length was 6.9 μm (11,300 subunits (16)), and although the reaction is too fast to measure the true initial rate, a lower limit equal to 16% s^{-1} can be estimated from the observed 80% disassembly in 5 s in the reaction which was diluted 2,500-fold. This is a lower limit for the initial rate, since the rate progressively decreases as the number concentration of microtubule ends falls as a result of the quantitative disassembly of the shorter microtubules. Based on the 16% s^{-1} value, the molecular rate constant for microtubule disassembly is at least (0.16) (11,300) = 1,800 subunits/microtubule/s. This value is about 15 times that which we previously observed for MAP-containing microtubules (17), but only about 5 times that determined with MAP-free microtubules diluted 80-fold (18). In the latter reaction, the disassembly rate is proportional to the extent of dilution (Fig. 5 in Ref. 18) so that the 350 s^{-1} value (17) may not represent a maximal rate. Karr *et al.* (19) have reported a rate equal to 210 s^{-1} for disassembly of MAP-free microtubules following a 20- to 50-fold dilution.

Reaction of Tubulin-GTP Subunits with Microtuble Ends

[3] The disassembly rate is about 40 times slower in the presence of 12% dimethyl sulfoxide (data not shown), which is expected since the critical concentration is about 10 times lower under these conditions.

TABLE II

Rate for dilution-induced disassembly of microtubules

Experiment	Dilution and diluent[a]	Microtubule remaining[b]		
		5 s	7 s	10 s
		%		
I				
a	25×	50 (45) [48]	28 (21)	11 (10) [19]
b	2500×	20 (11) [3.8]	4.6 (4.5)	2.5 (4.7) [3.5]
II				
a	25×	48 (49)		27.5 (32)
b	1000×	18 (15)		5.3 (5)
III				
a	25×, PFK, F6P, GDP	38 (50)	26 (30)	16 (26)
b	25×, GTP	23 (36)	9 (22)	3 (12)
IV				
a	25×, PFK, F6P, GDP	34	24	12
b	25×, GTP	25	14	5
c	2500×, GTP	11	5	0.4
V				
a	25×, PFK, F6P, GDP	53 (41)	40 (25)	36 (20)
b	25×, PFK, GTP	50 (44)	42 (33)	39 (24)
c	2500×, GTP	13 (5)	10 (2)	0.7 (0.6)

[a] Phosphofructokinase (PFK), fructose 6-phosphate (F6P), GTP, and GDP were present (where indicated) at concentrations of 22 units/ml, 0.75 mM, 200 μM, and 200 μM, respectively.

[b] A single batch of microtubules was used for the dilutions into different buffers (noted by a, b, and c) and results from replicate experiments are given in open type, parenthesis, and brackets. The effect on the rate of the magnitude of the dilution requires comparison of the results for a single experiment, since the microtubule length distribution is likely to be somewhat different in each experiment.

Containing GDP—Although the disassembly of microtubules goes to completion when steady-state microtubules are diluted 25-fold, which is expected since the tubulin subunit critical concentration is about 10 μM (see above) and the total tubulin in microtubules is only about 12 μM (Table I), the disassembly rate is significantly slower than when the same microtubules are diluted 1000 or 2500-fold (Fig. 1 and Table II, Experiments I, II, IV, b and c, and V, b and c). The slower rate for the less dilute reaction must result from reaction of the 0.054 μM TuT subunits (derived from the 25-fold dilution of the approximately 1.35 μM TuT critical concentration (Experiments IV and V in Table I) in the undiluted microtubules) with microtubule ends. An alternate possibility, that the rate is retarded by reaction of TuD subunits derived from subunits dissociating from the microtubules is ruled out since the disassembly rate following a 25-fold dilution of microtubules into a solution containing 10 μM TuD is the same as that in a control reaction without added protein (data not shown). Since 10 μM TuD does not influence the disassembly rate, the far lower concentration which can be derived from disassembly of the microtubules (100% disassembly of the microtubules would yield a TuD concentration of only 0.5 μM) cannot be responsible for the observed rate retardation. Furthermore, it was found in control reactions that with 10 μM TuD there is no measurable assembly of microtubules under these conditions (*i.e.* 0.5% dimethyl sufloxide) even when the reaction is nucleated with fragmented microtubules (data not shown). This confirms that TuD subunits are not reactive under the reaction conditions used here.

There remains the possibility that the rate retardation provided by the TuT subunits results from a reaction with microtubule ends containing GTP. GTP could possibly enter the microtubule ends by an exchange reaction (the κ'' reaction

in Fig. 1 of Ref.6). To evaluate this mechanism, we compared the rate following a 25-fold dilution into 200 μM GTP and into a mixture containing 200 μM GDP, as well as a GTPase mixture composed of phosphofructokinase and fructose 6-phosphate. This GTPase system was found to be sufficiently reactive so that under conditions identical to those in the dilution experiments, 0.5 μM [^{3}H]GTP (an amount about equivalent to that derived from the unbound nucleotide which is contained in the undiluted microtubule reaction mixture) was 70% converted to [^{3}H]GDP in 5 s. It was necessary that the GTPase system be especially reactive to overcome the effect of the acetate kinase in the microtubule mixture which would otherwise convert some of the 200 μM GDP in the reaction mixture to GTP. Thus, when steady-state microtubules were diluted 25-fold into the GTPase:GDP reaction mixture, the initial GDP/GTP ratio was about 400 (200 μM GDP/0.5 μM GTP) and this ratio progressively increased with time. As a result it is expected, based upon the only 3-fold higher affinity of tubulin subunits for GTP[4] as compared to GDP (20), that microtubule ends will not contain significant amounts of GTP. Consequently, if TuT subunits do not react with microtubule ends containing GDP, then the disassembly rate will be faster in the presence of GDP than with GTP. We found, however, that the disassembly is actually somewhat slower when the diluent contains GDP and the phosphofructokinase GTPase system (compare reactions a and b in Experiments III and IV in Table II). The decreased rate seen in the GTPase:GDP reaction mixture apparently results from the presence of the high concentration of phosphofructokinase (0.1 mg/ml). This is indicated by the fact that the rates were the same with a GDP and a GTP diluent, when phosphofructokinase was included in the GTP reaction (compare reactions a and b in Experiment V in Table II). It is possible that the enzyme binds to the microtubules so that the subunit dissociation rate is reduced.

DISCUSSION

It was initially believed that *in vitro* microtubule assembly follows the Oosawa-Kasai condensation model (22) in which the subunit flux rate (positive and negative) is linearly dependent on the tubulin subunit concentration. More recent experimental studies (18, 23) have revealed, however, that the tubulin subunit concentration dependence of the subunit flux rate is biphasic, so that the negative flux (*i.e.* disassembly) rates at tubulin subunit concentrations which are below the critical concentration are greater than predicted from a linear extrapolation of the rates observed at subunit concentrations which exceed the critical concentration. This increased dependence of the flux rate on the subunit concentration at low concentrations can be described as a "catastrophe" (24), in the sense that as the subunit concentration is reduced below the critical concentration it appears as if subunits are suddenly unable to prevent microtubule disassembly (by reacting with microtubule ends). Two models have been proposed (6, 23) to account for the inability of subunits to support microtubule assembly under conditions where this concentration is low. In one of these (6), it is assumed that TuT subunits do not add to microtubule ends containing TuD subunits ($\alpha_{1D} = 0$); in the other (23), it is further assumed that slowly dissociating TuT subunits forming a cap at microtubule ends serve to stabilize an unstable core of TuD subunits (*i.e.* 662 $\alpha_{-1T} = \alpha_{2D}$). We have recently realized that the biphasic subunit flux plot (18, 23) admits to an additional interpretation for the catastrophe. That is, the transition to a decreased concentration dependence of the subunit flux under conditions where the flux rate is positive may correspond to a reduced reactivity of tubulin subunits at high subunit concentrations relative to that which exists at low subunit concentrations (the opposite of the interpretation described above). A model has been proposed (7) which embodies this alternative interpretation. The experiments described here were designed to determine the basis for the "catastrophic" behavior of microtubules which is manifested in the biphasic concentration dependence of the rate for subunit flux (18, 23).

Reaction of TuT Subunits with Microtubule Ends Containing TuD Subunits—We have confirmed the results of previous studies (18, 23) showing that the concentration dependence for subunit flux is biphasic (Fig. 2). Although no significant positive subunit flux was observed under these conditions, the flux (assembly) rate at a high subunit concentration (10 μM) is not as rapid as predicted from a linear extrapolation of the observed flux rates at low subunit concentrations.

As noted above, the biphasic dependence of the flux rate on the subunit concentration has been accounted for (6) by assuming that TuT subunits are unable to react with microtubule ends containing TuD subunits (*i.e.* $\alpha_{1D} = 0$). According to this mechanism, concentrations of TuT subunits which are below the critical concentrations are unreactive because under conditions of net microtubule disassembly there is an increased probability that microtubule ends will be derived from the TuD subunit which constitute the body of the microtubule. As a result, there is a decreased fraction of ends which are available to react with the TuT subunits. This behavior has been described (6) in the statement that, "a significant initial slope in $J_\alpha(C)$ (*i.e.* flux dependence on subunit concentrations) can occur only if there is an appreciable value of κ'' ($\alpha_{1T}C$ term) or of α_{1D} (α_{1D} C term) or both." This will be the case since (6), "These are the transitions which make it possible for T from solution to attach to the polymer ends when C is very small." The effect of a nonzero value for α_{1D} is illustrated

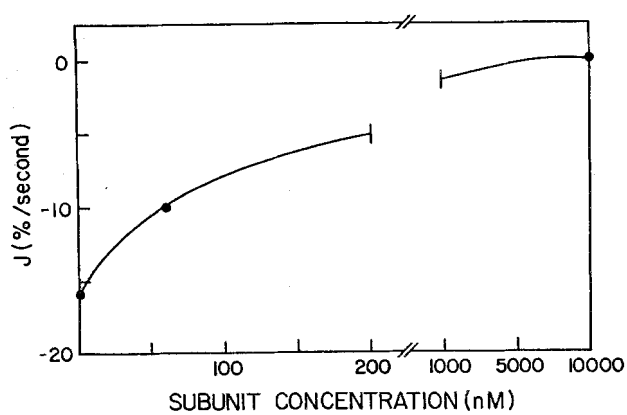

FIG. 2. **Subunit flux rate as a function of tubulin subunit concentration.** The data points for the two lowest concentrations are from the results in Fig. 1 and the critical concentration (J = 0) is estimated to be 10 μM (see text). The *solid line* was calculated from: $J = (C_1\alpha_1 - \alpha_{-1})/(1 + (C_1\alpha_1'/\alpha_{-1}'))$ (Equation 18 in Ref. 7), with $\alpha_1 = 1.6 \times 10^6\%\ M^{-1}\ s^{-1}$, $\alpha^{-1} = 1.6 \times 10^1\%\ s^{-1}$, $\alpha_1' = 1.0 \times 10^7\%\ M^{-1}\ s^{-1}$, and $\alpha_{-1}' = 1\%\ s^{-1}$. The primed rate constants are for nonproductive paths corresponding to the reactions defined in Footnote 2.

[4] Although our mechanism does not include GDP exchange into microtubules (see below and Ref. 9), the logic of this analysis requires that we consider these results in terms of the previously proposed mechanism. That is, when the observed GDP inhibition of the microtubule's steady-state GTPase rate (Fig. 6 in Ref. 21) was analyzed in terms of a mechanism in which nucleotides in the microtubule's terminal subunits are freely exchangeable, it was concluded (6) that the relative affinity for GTP and GDP is equivalent for dimeric tubulin subunits and for subunits at microtubule ends.

in Fig. 2 of Ref. 25, where it is shown that $J_\alpha(C)$ approaches linearity at all subunit concentrations when TuT subunit addition on ends containing TuD is only 40% as rapid as at ends containing TuT ($\alpha_{1D} = 0.4\alpha_{1T}$).

We find (Fig. 2) a significant initial slope in $J_\alpha(C)$ under conditions where there is no significant unbound GTP, so that nucleotide exchange into terminal subunits (the κ'' path) is insignificant. Thus, the first of the above-mentioned two possible explanations for a significant initial slope in $J_\alpha(C)$ (6) cannot account for the results. We have further demonstrated that the reactive species which is responsible for retarding the disassembly rate is TuT. From this we conclude that an α_{1D} term is appreciable, *i.e.* that TuT subunits readily react with microtubule ends containing TuD subunits. Thus, the assumption (6, 23) that the biphasic dependence of the flux rate on the tubulin subunit concentration results from $\alpha_{1D} = 0$ is incorrect.

Concerning the Nonlinear Dependence of the Flux Rate on the Subunit Concentration—We have proposed that TuT subunits are able to add to microtubule ends productively and nonproductively (7). The nonproductive binding hypothesis was derived from studies with enzymes (26, 27) to account for the fact that nonspecific substrates are frequently found to be unreactive (small V_{max}) but tightly bound (small K_m). Relating this to the microtubule assembly reaction, we presume that the net assembly reaction at subunit concentrations which exceed the critical concentration is relatively slow (low "V_{max}"), as a result of nonproductive subunit addition, but low concentrations of subunits are highly reactive in preventing disassembly (a small "K_m") for the same reason. Precedent for the presumed mechanism comes from the observation that TuD (8) and tubulin-colchicine (28) and tubulin-podophyllotoxin (29, 30) subunits add to microtubule ends in a way such as to preclude subsequent net microtubule elongation. Nonproductive addition of TuT subunits is assumed to follow a similar path. This process may reflect an alternative reaction path for a uniform population of TuT subunits or a unique path for a subspecies of the TuT population (a tubulin-colchicine-like subunit). These alternatives have not yet been differentiated.

The results obtained here can be quantitatively accounted for in terms of a proposed mechanism (*solid line* in Fig. 2), assuming rate constants such that nonproductive subunit addition is more favorable than the productive reaction (*i.e.* $\alpha_1/\alpha_{-1} = 10^5$, $\alpha_1'/\alpha_{-1}' = 10^7$). However, we consider these rate constants only rough approximations, since the relatively simple model which they describe (Fig. 1 in Ref. 7) does not take into account differences in productive and nonproductive TuT subunit addition at microtubule ends containing TuT and TuD subunits. If the nonproductive reaction is insignificant for ends containing subunits with GTP, then the positive flux rate will not reach a limiting value at high subunit concentrations.[5] The negative flux rate will, however, be nonlinearly dependent on the tubulin-GTP subunit concentration, as is observed, and the intercept on the abscissa is not expected to simply reflect the α_1/α_{-1} reaction, as is the case for the simpler model analyzed in Fig. 2.

Conclusions—We have demonstrated (Fig. 1) that TuT subunits react with microtubule ends with subunits containing GDP (*i.e.* $\alpha_{1D} \neq 0$). The rate for the α_{1D} reaction is nonlinearly dependent on the TuT subunit concentration (Fig. 2). The latter behavior is consistent with a mechanism in which TuT subunits add productively and nonproductively to microtubule ends (7). An alternate mechanism accounting for the nonlinear dependence of the negative flux rate on the subunit concentration, based upon an assumption that $\alpha_{1D} = 0$ (6), is ruled out. Thus, a mechanism based upon α_{1D} being equal to zero cannot serve as the basis for the existence of independent populations of growing and shrinking microtubules, as has been suggested (1). It should be noted the nonproductive subunit addition mechanism proposed here cannot account for the coexistence of independent populations of shrinking and growing microtubules (1).

[5] Although it has been found that the flux reaches a limiting rate at high concentrations (31, 32) and is nonlinearly dependent on the subunit concentration at concentrations which are below the critical concentration (18, 23), this is not always the case (see Fig. 12 in Ref. 33). This may reflect the fact that under some reaction conditions nonproductive TuT subunit addition does not occur to ends containing subunits with GTP or with GDP. Also, the wide variability in the rate for net microtubule assembly which has been observed in different laboratories may reflect the fact that the significance for nonproductive binding to ends containing TuT subunits varies with different tubulin preparations and under slightly different conditions.

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